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# Keep oxygen in check: contrasting effects of short-term aeration on hydrolytic versus oxidative enzymes in paddy soils

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**Abstract**: Enzymes produced by microorganisms and plants are very sensitive to variations in soil microclimate, yet most enzyme assays are conducted under oxic conditions irrespective of the origin of environmental samples. It remains unclear how short-term aeration (minutes to hours) affects the hydrolytic and oxidative enzymes in anoxic systems. This key gap in current methods was addressed by measuring the kinetics of hydrolytic phosphomonoesterase, β-glucosidase, and leucine aminopeptidase and the activities of oxidative phenol oxidases and peroxidases by fluorogenic substrates under oxic ($+O_2$) and anoxic conditions ($–O_2$). Aeration effects were tested in a flooded paddy soil with growing rice (research task 1: moderate $O_2$ limitation) and without rice (research task 2: strong $O_2$ limitation). We tested two hypotheses explaining possible effects of short-term aeration on hydrolytic versus oxidative enzymes. (1) Aeration promotes Fe(II) oxidation, which leads to the accumulation of phenolics through the "iron-gate" mechanism, thus suppressing the activities of hydrolytic enzymes compared to the anoxic conditions. (2) Aeration stimulates phenol oxidases that degrade phenolics according to the "enzyme latch" concept, thus eliminating the suppression of hydrolytic enzymes. The activities of hydrolytic enzymes were lower by 5–43% in both experiments under $+O_2$ compared to $–O_2$. In contrast, the activities of peroxidases and phenol oxidases were 2 to 14 times higher under $+O_2$ than under $–O_2$. Thus, the activation of oxidative enzymes under $+O_2$ was uncoupled from the hydrolytic activities. This contradicts both the "iron gate" and the "enzyme latch" mechanisms. We explain the short-term suppressive effect of $O_2$ in assays by increased concentrations of reactive oxygen

species, which decreased microbial activity. We conclude that our modification of enzyme assays under anoxic conditions is required for samples taken from low-oxygen environments to avoid underestimation due to rapid suppression of hydrolytic enzyme activities by $O_2$.

**Keywords:** anoxic conditions; suppression by oxygen; paddy soil; phosphomonoesterase; β-glucosidase; leucine aminopeptidase

# 1. Introduction

A large variety of biogeochemical processes in soil is mediated by enzymes, produced mainly by microorganisms, plant roots, and fauna (Kunito et al., 2018; Wang et al., 2021). Hydrolytic enzymes such as β-glucosidase, phosphatase, and leucine aminopeptidase catalyze the decomposition of organic polymers in soils to cover the demand by plants and microorganisms for carbon (C), phosphorus (P), and nitrogen (N), respectively (Alexander, 1977; Cosgrove, 1980; Hanson and Frohne, 1976). In contrast, oxidative enzymes such as phenol oxidases and peroxidases are expressed for a variety of purposes including ontogeny, defense, and the acquisition of C and nutrients from recalcitrant organic matter pool (Ladd, 1978; Sinsabaugh, 2010). Independent of soil sample origin, enzymatic activity assays are commonly performed under aerobic conditions (Wei et al., 2019; Parvin et al., 2018; Keiluweit et al., 2017), albeit oxygen ($O_2$) is a known suppressor for putative anaerobic microorganisms

59 (Dellwig et al., 2012). The prevailing paradigm claims that $O_2$ and hydrolytic
60 enzymes are decoupled at a biochemical level, so that the respective enzyme activities
61 are commonly measured under oxic conditions (Huang et al., 2021; Wei et al., 2019;
62 Li et al., 2019a; Peacock et al., 2015). Moreover, the terrestrial ecosystem models and
63 the mechanisms controlling the transformation of soil organic matter (SOM) have
64 been explored predominately under oxic conditions; the anoxic legacy effect is
65 existing, yet, even within well-drained soil systems but is largely overlooked in
66 previous studies (Keiluweit et al., 2017).

67 A general concept of the long-term effects of fluctuating oxic and anoxic
68 conditions on enzyme activities in soils has been described in the "enzyme latch"
69 hypothesis proposed by Freeman et al. (2001). It postulates that increased phenol
70 oxidase activity under $O_2$ exposure leads to the degradation of phenolics, which
71 inhibit hydrolytic enzyme activities, thereby stimulating SOM mineralization. Positive
72 relationships between $O_2$ availability and organic carbon mineralization rate (Waldrop
73 et al., 2004; Keiluweit et al., 2017) have also been reported for the upland ecosystems.
74 In contrast, reduced $O_2$ availability in humid tropical forest soils did not limit the
75 activity of hydrolytic enzymes, which was higher under anoxic vs. oxic conditions
76 (Hall et al., 2014). As opposed to oxidoreductases (e.g., phenol oxidase or peroxidase),
77 the functioning of soil hydrolytic enzymes does not require $O_2$ (Hall et al., 2014). In
78 addition to the "enzyme latch" concept, the "iron gate" paradigm proposes that the
79 oxidation of ferrous iron (Fe(II)) and C-complexation by ferric iron (Fe(III)) are the
80 main protective mechanisms against C loss in wetlands under $O_2$ exposure (Wang et

81 al., 2017). This in turn was attributed to the accumulation of phenolics caused by the
82 decreasing Fe(II) contents with aeration, as Fe(II) oxidation inhibits the oxidative
83 activity of phenols and promotes Fe-lignin phenol association. Thus, the “enzyme
84 latch” and “iron gate” may simultaneously control phenol oxidative activity and C
85 loss rates under $O_2$ exposure in peatlands, but it depends on the trade-off between $O_2$
86 and Fe(II) (Wen et al., 2019). Whether the mechanisms described require time (weeks
87 to months) to have clear effects on enzymatic reactions, or whether a short-term $O_2$
88 exposure of environmental samples adapted to anoxic conditions to $O_2$ can elicit a
89 rapid specific response in hydrolytic enzyme assays, remains uncertain. This calls for
90 evaluating the enzyme activities of samples from semi- or fully anoxic environments,
91 such as flooded rice paddy soils, and investigating how the measurement conditions
92 may influence enzyme activities in such ecosystems.

93 To address these apparent knowledge gaps, we adapted a commonly used
94 enzyme activity assay based on fluorogenically labeled substrates (Marx et al., 2001)
95 to the anoxic conditions by means of a portable glovebox. We applied the
96 Michaelis-Menten kinetic approach to calculate the maximal rate of an enzymatic
97 reaction ($V_{max}$) and the Michaelis constant ($K_m$) for three hydrolytic enzymes
98 contributing to C, N, and P turnover. The activities were measured at three dates of
99 rice plants growth (research task 1: moderate $O_2$ limitation due to diffusion from the
100 atmosphere and in the rhizosphere) and in combination with hydrolytic, two oxidative
101 extracellular enzymes measured in the same Fe-rich paddy soil without growing rice
102 (research task 2: strong $O_2$ limitation). The key aim of the study was to evaluate

whether enzyme activities in flooded paddy soils differ under anoxic and oxic short-term (45–150 min) assay conditions. Based on the proposed “enzyme latch” and “iron-gate” concepts, we hypothesized that short-term aeration (minutes to hours) either (1) stimulates the oxidative enzymes that degrade phenolics according to the “enzyme latch” concept, thus reinforcing hydrolytic enzymes, or (2) promotes Fe(II) oxidation, which leads to the accumulation of phenolics through the “iron-gate” mechanism, thus suppressing the activities of hydrolytic enzymes compared to the anoxic control. The following additional research questions were addressed: would the expected effects of short-term aeration on enzyme activities be modified with (1) a spatial natural aeration gradient from top via rooted to bottom soil in a rhizobox, and (2) a temporal natural aeration gradient arising from the age of the rice plants, which contributes to soil aeration via their aerenchyma as root biomass increases but also provide more C through higher exudation.

## 2. Materials and methods

### 2.1. Soil description

The soil was collected from the 0–20 cm depth in a paddy rice field at the Changsha Agricultural and Environmental Monitoring Station, Hunan Province, China (113°19′52″ E, 28°33′04″ N). The main soil physicochemical properties were pH 6.2, soil organic C 13.1 g $kg^{-1}$, total N 1.4 g $kg^{-1}$, available N 18.0 mg $kg^{-1}$, total P 0.3 g $kg^{-1}$, Olsen-P 3.7 mg $kg^{-1}$, and total Fe 15.7 g $kg^{-1}$ (Zhu et al., 2018). The soil

124 was passed through a 2 mm sieve and homogenized.

125

126 **2.2. Research task 1: moderate $O_2$ limitation**

127 *2.2.1. Experimental setup*

128 For each of the three enzyme assays, three PVC-rhizoboxes (9 in total) with
129 inner dimensions of 20.5 × 24.0 × 1.5 cm closed with a transparent, removable
130 plexiglas front cover were set as replicates. Rhizoboxes were specially constructed to
131 be water-tight using rubber sealing and screw-holders. One 20-day-old rice seedling
132 (*Oryza sativa* L. 'Two-line hybrid rice Zhongzao 39') was transplanted into a
133 rhizobox prefilled with water-saturated soil. After transplanting, all rhizoboxes were
134 adjusted with deionized water ca. 2 cm above the soil surface and the water level was
135 maintained throughout the experiment (except the dates of soil sampling; see section
136 2.2. below). Despite of flooding, moderate $O_2$ limitation in the Experiment 1 was
137 achieved through the diffusion of air to the water and topsoil as well as through
138 releasing of $O_2$ to the rhizosphere soil via aerenchimatous rice roots (Larsen et al.,
139 2015). All seedlings were grown in a climate chamber (KBF-S 720, Binder GmbH,
140 Tuttlingen, Germany) with 28 ± 1 °C day temperature and 24 ± 1 °C night
141 temperature, 70% relative humidity, and 12-h photoperiod. After transplanting, 30 mg
142 N as urea, 25 mg K and 20 mg P as $KH_2PO_4$ per kg dry soil were added to each
143 rhizobox as background fertilizers.

144 *2.2.2. Soil sampling*

145 Soil was collected at three consecutive dates of rice growth, i.e. after 10, 16–20,
146 and 28–31 days from rice transplanting. These ages of rice plants roughly
147 corresponded to a seedling (with 1 tiller), early (4 ± 1) and late (6 ± 1) tillering stages
148 of the vegetative phase of rice growth. Two days before each sampling date, the soil in
149 rhizoboxes was preconditioned by draining the flooding water to omit the loss at the
150 moment of opening. To maintain anoxic conditions in the moist soil, all the
151 rhizoboxes were opened inside a portable PVC glovebox (Captair® Pyramid
152 Glovebox 3015-00, Erlab DFS, Saint-Maurice, France) evacuated with a vacuum
153 pump (Ilmvac MP 301 Vp, Ilmvac GmbH, Ilmenau, Germany) and then back-flushed
154 with nitrogen to $O_2$ concentrations lower than 0.2%. The $O_2$ concentrations were
155 determined with an $O_2$-sensor (Greisinger GOX 100, GHM Messtechnik GmbH,
156 Remscheid, Germany).

157 After opening a rhizobox, the soil was collected from three compartments
158 roughly reflecting the gradient of natural aeration from higher to lower: top bulk (2–5
159 cm), rooted (5–15 cm), and bottom bulk (15–18 cm) (Figure 1a). In each compartment,
160 soil was collected from three random locations and then mixed into one sample of ca.
161 0.5 g moist weight. The sampling per compartment was repeated two times in the
162 glovebox – for oxic and anoxic assay – and the collected soil was placed into two 100
163 ml Kimble KIMAX borosilicate laboratory glass bottles (Kimble Chase Life Science
164 and Research Products, LLC., Meiningen, Germany), respectively. Depending on the
165 aeration treatment, either $N_2$-bubbled (anoxic) or normal deionized sterile water (with
166 dissolved $O_2$) was added to the respective bottles with soils at a soil-water ratio of

167 1:100. Before opening the glovebox, the bottles for the anoxic assay were tightly
168 sealed with thick air-impermeable butyl rubber septa. The glovebox was opened and
169 air filled the headspace of oxic bottles. Thereafter, the anoxic treatment was
170 additionally flushed with $N_2$ for 30 min. At the same time, the oxic treatment
171 remained open without additional manipulations. After slaking the soil, the oxic
172 bottles were closed with butyl septa and the suspension was prepared in oxic and
173 anoxic bottles simultaneously by shaking on a rotator (200 rpm) for 30 min before the
174 enzyme activity assays. A preliminary experiment has shown that the contribution of
175 enzymes bound to soil particles to the total enzyme activity is not significant after a
176 mild sonication (De Cesare et al., 2000) compared to a 30-min shaking without
177 sonication (data not shown). This suggested that the 30-min shaking was sufficient to
178 detect the majority of extracellular enzymes, both those released by active cells and
179 those enzymes stabilized on soil particles and colloids.

180 *2.2.3. Hydrolytic enzyme assays*

181 The activities of phosphomonoesterase (PME), β-glucosidase (BG), and leucine
182 aminopeptidase (LAP) were measured in independent variants (3 rhizoboxes per
183 enzyme) using fluorogenically labelled substrates of 4-methylumbelliferyl-phosphate,
184 4-methylumbelliferyl-β-D-glucoside, and L-leucine-7-amino-4-methylcoumarin
185 hydrochloride (all substrates were purchased from Sigma-Aldrich Co. Ltd),
186 respectively, according to the established method (Marx et al., 2001). We measured
187 enzyme kinetics according to the Michaelis-Menten approach with a saturating range
188 of substrate concentrations: 0, 5, 10, 20, 50, 100, 150, and 200 μM. Substrate and

189 buffer (see below) were prepared in duplicate, one replicate was flushed with $N_2$ for
190 20 min and then used for enzyme determination under anoxic conditions in the
191 glovebox (–$O_2$ assay) and the other replicate without any pretreatment was used for
192 enzyme determination under oxic conditions (+$O_2$ assay). For both assay treatments,
193 50 µl soil suspension, 100 µl 4-methylumbelliferone (MUF) or
194 7-amino-4-methylcoumarin (AMC)-based substrate, and 50 µl MES or TRIZMA
195 buffer were added into a 96-well black microplate (Brand GmbH, Wertheim,
196 Germany). After addition, microplates were incubated for enzymatic reaction
197 development. To maintain anoxic conditions during incubation, anoxic microplates
198 were prepared in the glovebox with $O_2$ concentrations lower than 0.2% from the
199 atmospheric level. For +$O_2$ assay, the preparation and incubation of microplates were
200 done outside the glovebox. The difference in enzyme kinetic parameters between oxic
201 and anoxic assays was termed as “aeration effect”. Pre-tests were made to define a
202 period of time between 0 and 120 min after addition of substrates ensuring linear
203 development of fluorescent signal. So, the fluorescence was measured at 30 and 60
204 min after addition of substrates to soil on a Victor 1420-050 Multi label counter
205 (PerkinElmer, USA) using a protocol with the excitation and emission wavelengths at
206 355 nm and 460 nm, respectively. The duration of reading per well was 0.1 s, so full
207 measuring of microplates was completed within 10 s. The effect of such a short period
208 of time compared with the total time of exposure to $O_2$ for the oxic assay including
209 suspension preparation (ca. 2 h) was assumed to be negligible during anoxic assays.
210 The total time of exposure to $O_2$ for the oxic assay including suspension preparation

211 was ca. 2 h.

212

### 213 2.3. Research task 2: strong $O_2$ limitation

214 *2.3.1. Experimental setup*

215 Strong $O_2$ limitation in the paddy soil was tested in an incubation experiment
216 without rice plants. The research task 2 comprised two objectives: (1) to reveal the
217 earliest effect of aeration on enzyme activities by measuring the signal during the first
218 45 min incubation of soil and substrates, (2) to estimate the aeration effects on the
219 activities of phenol oxidases and peroxidases and to correlate the activities with the
220 concentrations of Fe(II) and Fe(III) in soil suspensions of oxic and anoxic assays.

221 Same paddy soil was used for the research tasks 1 and 2 (see section 2.1.). Four
222 100 ml Kimble KIMAX borosilicate laboratory glass bottles (Kimble Chase Life
223 Science and Research Products, LLC., Meiningen, Germany) were filled with 20 g
224 (dry weight) water-saturated soil each (Figure 1b). Additionally, 10 ml deionized
225 water was added to each bottle. The soil was pre-incubated anaerobically in the dark
226 for 10 days at 25 °C in a climate chamber (KBF-S 720, Binder GmbH, Tuttlingen,
227 Germany) to establish strong anoxic conditions.

228 *2.3.2. Soil sampling*

229 After incubation, all the bottles were opened inside the glovebox as explained
230 above (section 2.2.2.). Soil in each bottle was stirred with a spoon and two

231 subsamples ca. 0.5 g moist soil were collected individually for oxic and anoxic assays,
232 respectively. The soil suspension was prepared as described above (section 2.2.2.).

233 *2.3.3. Hydrolytic enzyme assays*

234 Same hydrolytic enzymes – PME, BG, and LAP – were assayed as described
235 above (see 2.2.3. section). To reveal the earliest effect of aeration on enzyme activities,
236 the fluorescence was measured at 0, 15, 30, 60, 90, and 120 min after the addition of
237 substrates to microplates under oxic and anoxic conditions.

238 *2.3.4. Phenol oxidase and peroxidase activity assays*

239 Phenol oxidase activity and peroxidase activity were measured using a substrate
240 Amplex Red (10-acety-3,7-dihydroxyphenoxazine, purchased from Sigma-Aldrich Co.
241 Ltd), according to Khosrozadeh et al. (2022). Briefly, 1 mg Amplex Red was
242 dissolved in 300 µl dimethyl sulfoxide (DMSO, Zhou et al., 1997), and then TRIZMA
243 buffer was added to obtain a final concentration of 500 µM. The Amplex Red solution
244 was prepared in duplicate and was flushed with $N_2$ for 5 min. For both assay
245 treatments (anoxic and oxic), 50 µl soil suspension, 100 µl Amplex Red solution, and
246 50 µl TRIZMA buffer were added into a 96-well black microplate (Brand GmbH,
247 Wertheim, Germany). To distinguish peroxidase activity from phenol oxidase in total
248 oxidative enzymatic reaction with Amplex Red, 10 µl of 0.3% $H_2O_2$ were added to
249 each well of a separate microplate after the soil suspension, Amplex Red solution, and
250 TRIZMA buffer were added as described above. The anoxic conditions during
251 respective assay, including the incubation period of soil and substrate in microplates,

252 were maintained using the glovebox. All manipulations of the oxic treatment were
253 conducted in a similar way but under the room conditions. The fluorescence was
254 measured at 0, 15, 30, 60, 90, and 120 min after the addition of substrates to
255 microplates on a TECAN Infinite 200® PRO (Tecan Austria GmbH, Austria) using a
256 protocol with the excitation and emission wavelengths at 530 nm and 585 nm,
257 respectively.

258 *2.3.5. Fe(II) and Fe(III) concentration measurement*

259 Determination of Fe(II) and Fe(III) concentrations in oxic and anoxic soil
260 suspensions was done according to Elrod et al. (1991). Before analysis, all soil
261 suspensions were filtered through a filter paper (Whatman No. 42). For oxic assay, 2
262 ml filtrate were mixed with 500 µl ammonium acetate buffer (pH 4.5) and 500 µl
263 1,10-phenanthroline solution (0.5%) in a transparent cuvette (Th. Geyer GmbH & Co.
264 KG, Renningen, Germany) and then measured at 512 nm on a spectrophotometer
265 (NanoPhotometer® NP80, Implen GmbH, Munich, Germany). Then, 200 µl ascorbic
266 acid solution (10%) was added to cuvettes to completely reduce Fe(III) to Fe(II). After
267 30-min reaction, total Fe concentration was measured as described above. Fe(III)
268 concentration was calculated as the difference between total Fe and Fe(II)
269 concentrations. The measurement was repeated after 15, 30, 60, 90, and 120 min
270 incubation of soil suspensions. For anoxic assay, the described procedure was
271 conducted inside the glovebox and the measurement was conducted immediately after
272 the cuvettes were removed from the glovebox. Determination of Fe(II) and total Fe
273 was done on the same anoxic suspension but in separate runs to exclude effect of

274 aeration during 30 min Fe (III) reduction by ascorbic acid. Calibration was performed

275 with $FeCl_3$ at increasing concentrations of 0, 5, 10, 25, 50, 100, 200, and 300 µM.

276

277 **2.4. Enzyme kinetics**

278 To estimate the rate of hydrolytic enzyme activities in the research tasks 1 and 2,

279 the assays were calibrated using either MUF or AMC pure substances at increasing

280 concentrations of 0, 100, 200, 500, 800, and 1200 pmol well$^{-1}$ of a microplate. For

281 phenol oxidative and peroxidase activity estimation, the assays were calibrated using

282 resorufin at increasing concentrations of 0, 500, 1000, 1500, and 2000 nmol well$^{-1}$.

283 Calibration was conducted in parallel under oxic and anoxic conditions. All pure

284 substances were purchased from Sigma Aldrich Co. Ltd.

285 Based on the calibration, the rates of three hydrolytic enzyme activities were

286 calculated as nmol MUF or AMC per g soil on a dry weight basis per hour at each of

287 the substrate concentrations added (in µmol g$^{-1}$ dry soil). The Michaelis-Menten

288 equation was used to calculate the kinetic parameters $V_{max}$ and $K_m$ for each enzyme:

289 $$\upsilon = (V_{max} * S)/(K_m + S) \quad (1)$$

290 where $\upsilon$ is the reaction rate (nmol g$^{-1}$ soil h$^{-1}$), S is the substrate concentration, $V_{max}$ is

291 the maximum reaction rate of enzymatic activity at saturated substrate concentration

292 calculated from the increment of fluorescence values between the selected time

293 intervals (0–15, 15–30, 30–60, 60–90, and 90–120 min), and $K_m$ is the substrate

294 concentration at half-maximal rate ($^1/_2$ $V_{max}$). $V_{max}$ and $K_m$ were estimated using

295 non-linear curve fitting in GraphPad Prism 8 (GraphPad Software, Inc., San Diego,
296 USA). Oxidative enzymes were calculated based on a single saturating concentration
297 ($\upsilon = V_{max}$) from a linear regression of calibration and presented as nmol resorufin per
298 g soil on a dry weight basis per hour.

299

300 **2.5. Statistical analysis**

301 For the research task 1, a two-way ANOVA with repeated measures (rice growth
302 stage, n = 3) was used to test the effects of (i) assay condition ($+O_2$ vs. $–O_2$ assays)
303 and (ii) soil compartment on $V_{max}$ and $K_m$. For the research task 2, a two-way ANOVA
304 was used to test the effects of (i) assay condition ($+O_2$ vs. $–O_2$ assays) and (ii)
305 incubation time on $V_{max}$ and $K_m$ of hydrolytic enzymes, the activity of phenol
306 oxidases and peroxidases, and the concentration of Fe(II) and Fe(III) in soil
307 suspensions. Linear regression analysis was used to determine the relationships
308 between Fe(II) or Fe(III) concentrations and incubation time in soil suspensions. All
309 statistical tests were conducted using SPSS (Version 21, IBM, Armonk, NY, USA).

310

## 311 3. Results

312 **3.1. Calibration of assays under anoxic and oxic conditions**

313 Calibration curves of MUF (Figure S1a), AMC (Figure S1b), or resorufin (Figure
314 S1c) obtained under $–O_2$ and $+O_2$ demonstrated strong linearity ($r^2$~0.967–0.999) with

315 given concentrations. The slopes of the MUF and AMC calibration lines varied by
316 0.1% and 1.7% under $–O_2$ vs. $+O_2$ assays, respectively. The differences between the
317 slopes in $–O_2$ and $+O_2$ assays were not statistically significant for MUF, AMC, or
318 resorufin (Figure S1).

319

320 **3.2. Kinetic parameters of the hydrolytic enzymes**

321 *3.2.1. Research task 1: moderate $O_2$ limitation*

322 The activities of the three tested enzymes demonstrated the saturation pattern
323 with increasing substrate concentrations from 0 to 200 μmol $g^{-1}$ soil under both
324 aeration treatments (Figure S2, S3). $V_{max}$ was most strongly suppressed by $O_2$ for BG
325 (28–43%), followed by PME (12–27%) and LAP (9–22%) (Figure 2a, b, c). This was
326 more pronounced in rooted soil than in bulk soil (Figure 2a, b, c). The suppression
327 effect by $O_2$ demonstrated diverse patterns (increase, decrease, or no change) with rice
328 growth for all tested enzymes (Figure 2a, b, c). Compared with $V_{max}$, the affinity of
329 enzymes to substrates ($K_m$ values) was generally less affected by $O_2$. Only the affinity
330 of PME decreased (higher $K_m$) by 11–17% under $–O_2$ vs. $+O_2$ (Figure 2d). Between
331 the compartments, the $K_m$ values were overall higher in rooted vs. top bulk soil and
332 especially bottom bulk soil for all enzymes in both aeration assays (Figure 2d, e, f).
333 The substrate affinity dynamics of PME increased with rice growth, except for rooted
334 soil under $+O_2$ conditions (Figure 2, bottom row).

335 *3.2.2. Research task 2: strong $O_2$ limitation*

336 $V_{max}$ was suppressed by 6–26% for BG, 8–24% for PME, and 5–23% for LAP
337 under $+O_2$ vs. $–O_2$ (Figure 3a, b, c). The difference in $V_{max}$ between $+O_2$ vs. $–O_2$
338 increased from 5–8% to 20–24% with incubation time from 15 to 60 min and then
339 stabilized between 19–26% after 1-h incubation (Figure 3a, b, c). The increased
340 activities of all three enzymes at initial 15–30 min (for PME and BG) and up to 60
341 min (for LAP) was attributed to high autofluorescence of substrates (fluorescence
342 during solubilization of substrates which was not caused by an enzymatic reaction)
343 (Figure 3, red arrows). In contrast to $V_{max}$, the aeration had no effects on the affinity
344 of enzymes to substrates (Figure 3d, e, f), except for PME which affinity to the
345 substrate increased with incubation time (Figure 3d).

346

347 **3.3. The activity of phenol oxidases and peroxidases and the concentration of**
348 **Fe(II) and Fe(III) in the research task 2**

349 In contrast to hydrolytic enzymes, oxidative phenol oxidase and peroxidase
350 activities were up to 14 and 2 times higher under $+O_2$ than $–O_2$, respectively (Figure
351 4a). Fe(II) concentration in soil suspension gradually decreased at a rate of 0.02 μM
352 $min^{-1}$ under $+O_2$ assays during 150 min exposure to air (Figure 4b). In contrast, Fe(III)
353 concentrations gradually increased at a rate of 0.014 μM $min^{-1}$ under $+O_2$ assays
354 (Figure 4b).

355

356 ## 4. Discussion

357 **4.1. Effects of short-term aeration on hydrolytic enzymes, phenol oxidases, and**
358 **peroxidases**

359 Incubation strategies under moderate (research task 1) and strong $O_2$ limitation
360 (research task 2) both independently revealed the short-term suppressive effect of
361 aeration on hydrolytic enzyme activities in a paddy soil. So, in soils subjected to
362 moderate $O_2$ limitation, activities of hydrolytic enzymes were suppressed by 9–43%
363 by aeration (Figure 2a, b, c). Similarly, for soils incubated under strong $O_2$ limitation,
364 the maximal enzymatic reaction rate after a reduction of autofluorescence (from
365 60–90 min depending on an enzyme) decreased by 19–26% with aeration of the
366 enzyme assay (Figure 3a, b, c). This contradicted the first hypothesis that hydrolytic
367 enzyme activities would increase after the transition from anoxic to oxic conditions.
368 The decrease in $V_{max}$ by aeration in the assays was greater by 6–57% in rooted vs.
369 bulk soil under the moderate $O_2$ limitation. This answered our first research question
370 that the suppressive effects of short-term aeration on hydrolytic enzyme activities are
371 modified along natural aeration gradient. This means, the natural aeration gradient
372 (Lüdemann et al., 2000; Bai et al., 2015) in the paddy soil from top bulk through
373 rooted and down to bottom bulk soil could not alleviate the suppression of enzymes
374 by aeration.

375 Regarding the second research question, no clear patterns of $V_{max}$ by aeration of
376 the assays with rice growth were observed, suggesting that the suppressive effects of
377 short-term aeration on hydrolytic enzymes are independent of plant root biomass and
378 amount of provided exudates. The short-term aeration only affected the affinity of

379 phosphomonoesterase to substrates ($K_m$ values) (Figure 2d), indicating that the effects
380 of moderate $O_2$ limitation are enzyme-specific. Moreover, the phosphomonoesterase
381 $K_m$ values decreased with the duration of aeration under strong $O_2$ limitation (Figure
382 3d). This can be interpreted as (1) a selective suppression of less effective enzymatic
383 systems, and/or (2) the production of a set of isoenzymes of phosphomonoesterase
384 with the same function but different chemical structure resulting in higher affinity to
385 substrate under oxic conditions (Hochachka and Somero 2002). The latter can be in
386 turn interpreted as a quick feedback mechanism and physiological response of the
387 microorganisms to the decreasing phosphate availability in soil solution due to the
388 immobilization of phosphates on Fe(III) from Fe(II) oxidation under oxic conditions.

389 Long-term oxic conditions and the removal of phenolics have been suggested to
390 increase hydrolytic enzyme activities in paddy soils (Wang et al., 2022), wetlands
391 (Wang et al., 2017), and peatlands (Freeman et al., 2001). However, we found that
392 short-term (2.5 h) aeration during enzymatic assays under strong $O_2$ limitation had an
393 overall negative effect on $V_{max}$ values of the three tested hydrolytic enzymes in
394 naturally anoxic flooded paddy soil. In contrast, oxic conditions stimulated phenol
395 oxidase activity (Figure 4a) and may therefore suggest an increased removal rate of
396 phenolics. Thus, our findings could not support the proposed "enzyme latch"
397 mechanism, which states that the suppression of hydrolytic enzymes is associated
398 with decreased activity of oxidative enzymes and the accumulation of phenolics.
399 Moreover, the Fe(II) oxidation rate of 0.02 μM $min^{-1}$ observed in the present study
400 was lower than the values (0.1–0.5 μM $min^{-1}$) found in other studies on paddy soils

401 (Li et al., 2016; Li et al., 2019b). As a result, the production of Fe(III) at such a low
402 rate cannot strongly affect the phenolics stoichiometrically, thereby contradicting the
403 second hypothesis. This confirms the inability of "iron gate" to explain short-term
404 aeration effects. Therefore, neither "enzyme latch" nor "iron gate" can explain the
405 suppression of hydrolytic enzyme activities in the short-term.

406

407 **4.2. Mechanisms of the short-term effects of aeration on hydrolytic enzymes**

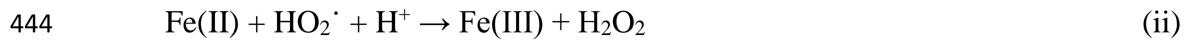
408 Enzyme activities are the net effect of complex processes including enzyme
409 production, stabilization, degradation, and inhibition (Allison, 2006). Below, we
410 propose three mechanisms, which in our view may most comprehensively explain the
411 observed short-term suppressive effect of $O_2$ on hydrolytic reactions (Figure 5):

412 (1) Abrupt aeration inhibited the activity of obligate anaerobic microorganisms
413 and initiated a shift in microbial metabolic pathways, restricting the secretion of *de*
414 *novo* formed enzymes. Generally, $O_2$ could affect microbial communities in two ways:
415 (i) long-term change in microbial community structure if redox conditions persist
416 longer than the average generation time of organisms, (ii) short-term change in the
417 activity and metabolic pathways of the community if the redox conditions persist
418 shorter than organisms' average generation times (Deangelis et al., 2010). Although
419 we did not determine the microbial community changes, there is evidence that such
420 changes are not essential within a timeframe of 2.5 h, given the 4–11 h time lag
421 preceding microbial growth and the 1.8–2.8 h generation time of growing

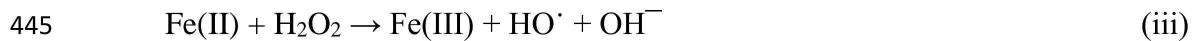
microorganisms in soil (Bååth, 1992; Blagodatskaya et al., 2009). Microbial biomass and community structure did also not change in a humid tropical soil with short-term redox fluctuation under alternating flushing of air and $N_2$ every 12 h (Pett-Ridge et al., 2006). Therefore, 2.5-h aeration in our both experiments should rather cause short-term changes in activity than shifts in microbial community structure (Figure 5b, pathway I). Facultative anaerobes and micro-aerophilic groups can adapt to common $O_2$ fluctuations (Yadav et al., 2014). However, the abrupt exposure of an established anoxic environment to air will most probably cause a direct suppressive effect on active anaerobic microorganisms and strongly reduce *de novo* enzymes synthesis. This was indirectly confirmed by the fact that the negative effect of aeration on hydrolytic enzyme activities increased with increasing duration of $O_2$ exposure, during which enzyme turnover and degradation continued but no new enzymes were supplied by the highly $O_2$-stressed microbial community (Figure 3).

(2) The reactive oxygen species (ROS), such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($HO^{\cdot}$), and hydroperoxyl ($HO_2^{\cdot}$), can be produced as a result of molecular $O_2$ reduction by Fe(II) and may directly suppress microorganisms after the transition from anoxic to oxic conditions (Fenchel and Finlay, 2010). Importantly, ROS could be generated quickly initially (Grant and Loake, 2000). For example, $H_2O_2$-mediated oxidative cross-linking of bean cells was initiated within 2 min (Bradley et al., 1992). ROS can be generated through Fenton and Fenton-like reactions (Hall and Silver, 2013):

$$Fe(II) + O_2 + H^+ \rightarrow Fe(III) + HO_2^{\cdot} \quad \text{(i)}$$

444 

$$Fe(II) + HO_2^{\cdot} + H^+ \rightarrow Fe(III) + H_2O_2 \quad \text{(ii)}$$

445 

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\cdot} + OH^- \quad \text{(iii)}$$

446 Although ROS species and their concentration dynamics were not measured in the
447 current experiment, estimates based on Fe(II) oxidation rate of 0.02 μM $min^{-1}$ confirm
448 the stoichiometric generation of 0.06 nmol of cumulative ROS in each well of a
449 microplate per hour. Stimulated oxidative enzyme activities, which use ROS such as
450 $H_2O_2$ as co-substrates, support the concept of increased ROS formation, which may be
451 responsible for exertion of a negative effect on microbial activities after an abrupt
452 aeration. Aerobes and facultative anaerobes possessed complex protective
453 mechanisms such as the production of catalase, superoxide dismutase, and other
454 compounds to reduce ROS (Fenchel and Finlay, 2010). However, these protective
455 enzymes are generally lacking or occur at very low concentrations in anaerobes
456 (Fenchel and Finlay, 1994). An abrupt aeration can therefore suppress the entire
457 metabolic activity of anaerobes, including the production of hydrolytic enzymes
458 (Figure 5b, pathway II), at least until sufficient agents are synthesized to protect cells
459 from ROS damages.

460 (3) Aerobes and facultative anaerobes may respond to oxidative stress caused by
461 ROS by redirecting their resources from the secretion of hydrolases to protection.
462 Microbial functions are prone to immediate changes, especially under conditions
463 where stress becomes intolerable (Tikariha et al., 2018). For example, the
464 ammonia-oxidizers (Bodelier et al., 1996) and the nitrifying bacterial community

465 (Jensen, 1993) in fresh water sediments resumed nitrification within 1 h upon
466 exposure to $O_2$. This and other examples (Kalia et al., 2011; Tan et al., 2014; Cabiscol
467 et al., 2000) confirm that the 2-h aeration likely exerted intolerable stress on the
468 microbial community and caused changes in enzyme kinetic parameters. To protect
469 themselves against oxidative stress, the compensatory mechanisms established by
470 microorganisms include production of catalase, superoxide dismutase, glutaredoxin,
471 and thioredoxin (Cabiscol et al., 2000). For example, manganese-containing
472 superoxide dismutases and hydroperoxidase I were produced by *Escherichia coli* in
473 response to oxidative stress (Compan and Touati, 1993; Finn and Condon, 1975).
474 Along with such a direct compensatory mechanism, the indirect effect will be driven
475 by the need of microorganisms to overuse their resources in dealing with the stress
476 (Schimel et al., 2007). Apparently, the production and release of enzymes by
477 microorganisms are costly, energy-consuming processes (Schimel and Weintraub,
478 2003). If this energy is devoted to compensatory production of catalases and
479 ferroxidases to resist oxidative stress, then there could be a concurrent decrease in
480 hydrolases production (Figure 5b, pathway III).

481 In summary, this study demonstrated for the first time the clear need to consider
482 *in situ* conditions for the soil enzyme assays. The proposed mechanisms should be
483 further proven by (i) in-depth verification based on ROS identification and
484 concentration measurements and (ii) the long-term effects of aeration on enzyme
485 kinetic parameters during the shift from anoxic to oxic conditions, e.g. after water
486 drainage or under the alternative wetting/drying rice cultivation management.

487

488 ## 5. Conclusions

489 We evaluated the effect of short-term aeration (for ca. 2–2.5 h) on activities of
490 three common hydrolytic enzymes catalyzing the decomposition of C-, N-, and
491 P-containing organic compounds in flooded paddy soil. Our study demonstrated for
492 the first time that measuring the enzyme kinetics in natively low-oxygen systems
493 under anoxic conditions e.g. in a glovebox is an essential methodological requirement
494 to assess enzymatic reaction rates and affinity to substrates of this oxygen-sensitive
495 biological soil feature. Overall, the potential activities ($V_{max}$) of phosphomonoesterase,
496 β-glucosidase, and leucine aminopeptidase in a paddy soil were underestimated under
497 oxic conditions by 5–43% as compared to anoxic conditions. In contrast, phenol
498 oxidases were up to 14 times higher and peroxidases 2 times higher after an abrupt
499 aeration. Thus, short-term (a few hours) aeration strongly affected the
500 enzymatically-mediated processes of enzymes produced under shortage of $O_2$. We
501 therefore suggest that enzymatic assays for anoxic (e.g. humid tropical soils, rice
502 paddies) and especially more strict anaerobic environments (e.g. wetlands, peatlands,
503 and sediments) should be conducted under controlled, $O_2$-free conditions. Moreover,
504 the underestimation of hydrolytic enzyme activities due to an aeration bias in enzyme
505 assays may lead to a strongly skewed mechanistic understanding of SOM
506 transformations in anoxic environments with follow-up complications for
507 process-based modeling.

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674 **Figure captions**

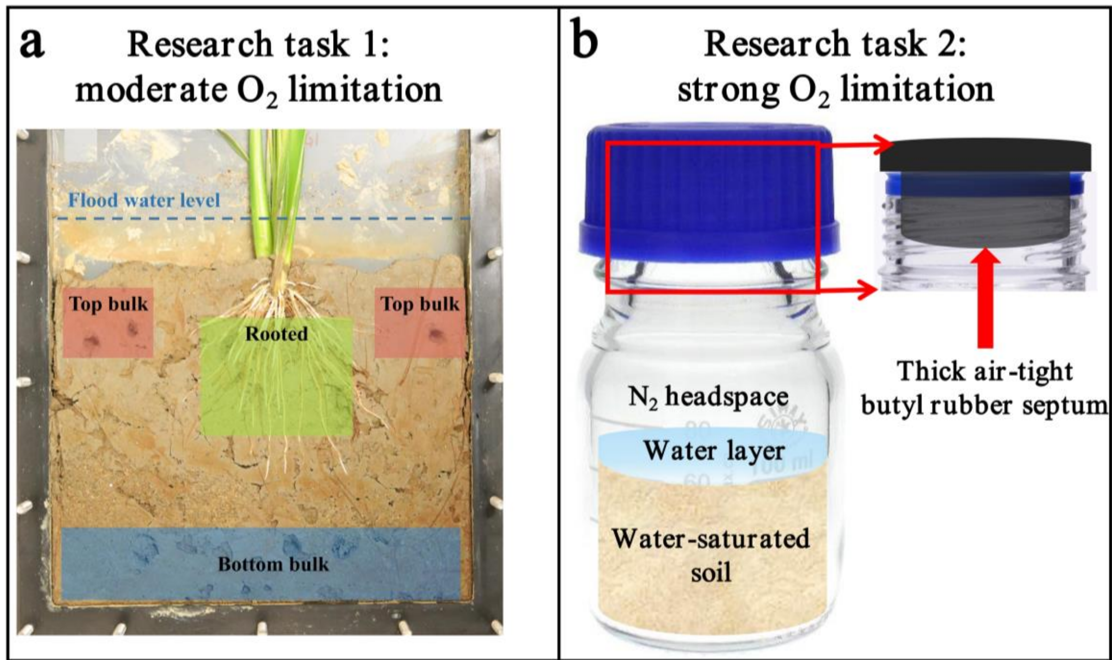
675 **Figure 1** (a) Sampling locations in the three soil compartments 48 hours after flood
676 water drainage for the Research task 1: moderate $O_2$ limitation; shaded spots within
677 each compartment correspond to the removed soil; the level of flooded water
678 maintained during experiment is shown schematically by a dashed line. (b) Incubation
679 set-up for the Research task 2: strong $O_2$ limitation with water-saturated soil in a
680 bottle sealed with a thick air-impermeable butyl rubber septum.

681 **Figure 2** The maximum reaction rate ($V_{max}$) of phosphomonoesterase (PME, a),
682 β-glucosidase (BG, b), and leucine aminopeptidase (LAP, c) and the affinity to a
683 substrate ($K_m$) of PME (d), BG (e), and LAP (f) at the top bulk, rooted, and bottom
684 bulk soil of rhizoboxes with growing rice under moderate $O_2$ limitation (research task
685 1) in oxic (+$O_2$, dashed lines) and anoxic (–$O_2$, solid lines) assays. The data are means
686 ± standard deviations (n = 3).

687 **Figure 3** The maximum reaction rate ($V_{max}$) of phosphomonoesterase (PME, a),
688 β-glucosidase (BG, b), and leucine aminopeptidase (LAP, c) and the affinity to a
689 substrate ($K_m$) of PME (d), BG (e), and LAP (f) in soils under strong $O_2$ limitation
690 (research task 2) in oxic (+$O_2$, dashed lines) and anoxic (–$O_2$, solid lines) assays. The
691 data are means ± standard deviations (n = 4). The vertical red lines correspond to the
692 duration of autofluorescence when enzyme activity is not measurable. Downward
693 arrows represent the negative aeration effect on $V_{max}$. Size of a arrow indicate the
694 relative intensity of the aeration.

695 **Figure 4** The activity (a) of phenol oxidases (yellow lines) and peroxidases (blue lines)
696 and the concentration dynamics (b) of soluble Fe(II) (blue) and Fe(III) (yellow) in soil
697 suspension either in oxic (+$O_2$) or anoxic (–$O_2$) assays. The data are means ± standard
698 deviations (n = 4). Solid lines in subfigure b indicate significant linear correlations.
699 Arrows represent the aeration effect on either the activity of phenol oxidases and
700 peroxidases or the concentration dynamics of soluble Fe(II) and Fe(III) in soil
701 suspension. Size of arrows indicates the relative intensity and direction corresponds to
702 a positive or a negative aeration effect on a parameter.

703 **Figure 5** Mechanisms of hydrolytic enzymes suppression by short-term (during 2
704 hours) $O_2$ exposure. Left side (a): enzymatic reactions conducted by hydrolytic
705 enzymes under anoxic conditions. Right side (b): reduction of microbial activity
706 (pathway I), toxicity of reactive oxygen species (ROS) to microbial cells (pathway II),
707 and compensatory mechanism of anti-stress enzyme production, e.g. catalase,
708 superoxide dismutase (pathway III). Size of arrows corresponds to relative intensity of
709 enzyme production or reaction rates. Triangles on top reflect the relative increase of
710 the short-term suppressive effect of oxygen (brown) and the concurrent relative
711 decrease of enzymatic maximal reaction rate ($V_{max}$, blue) with $O_2$.

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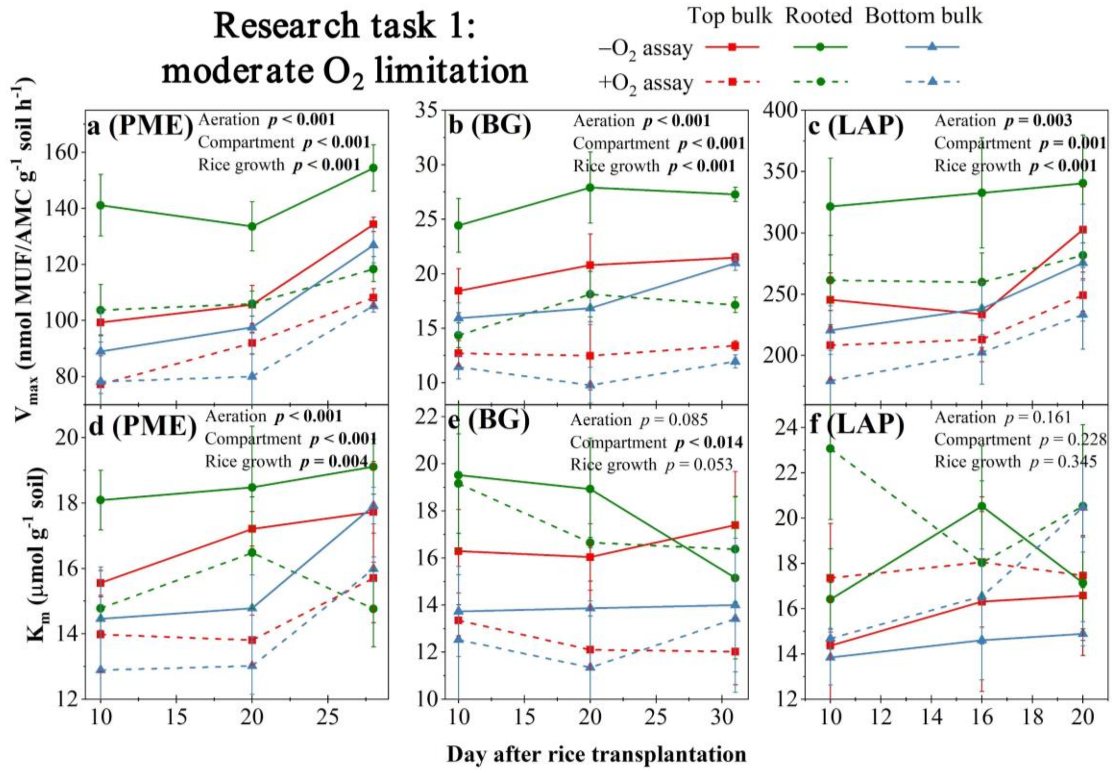
713 **Figure 1**

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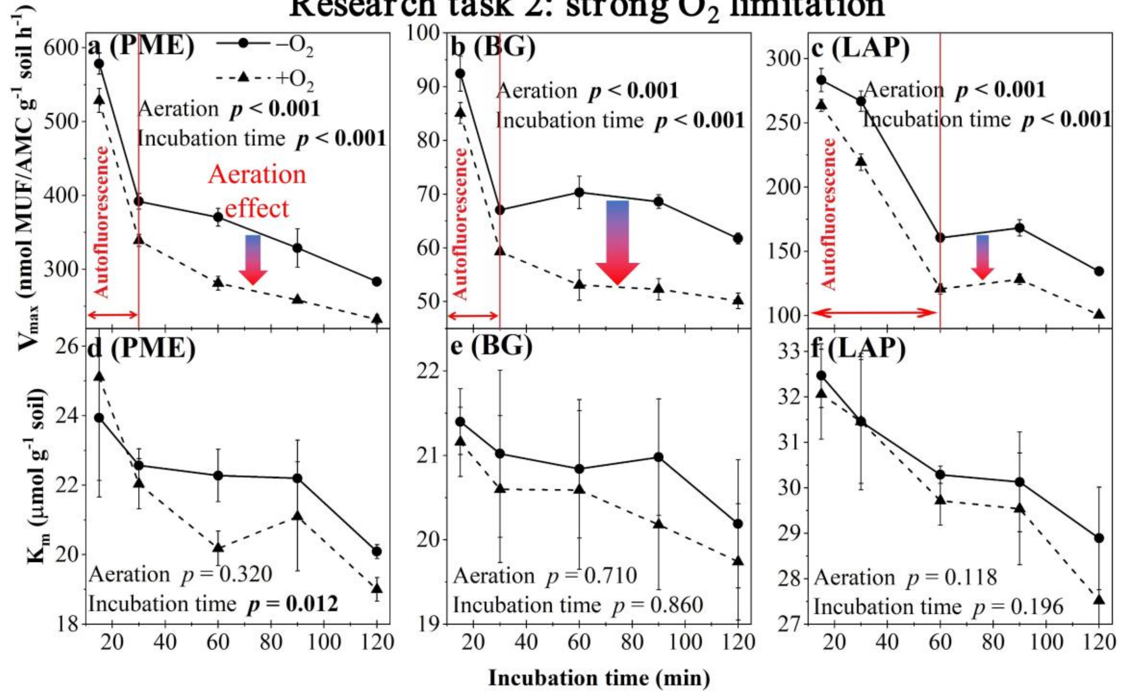
715 **Figure 2**

Research task 2: strong $O_2$ limitation

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717 **Figure 3**

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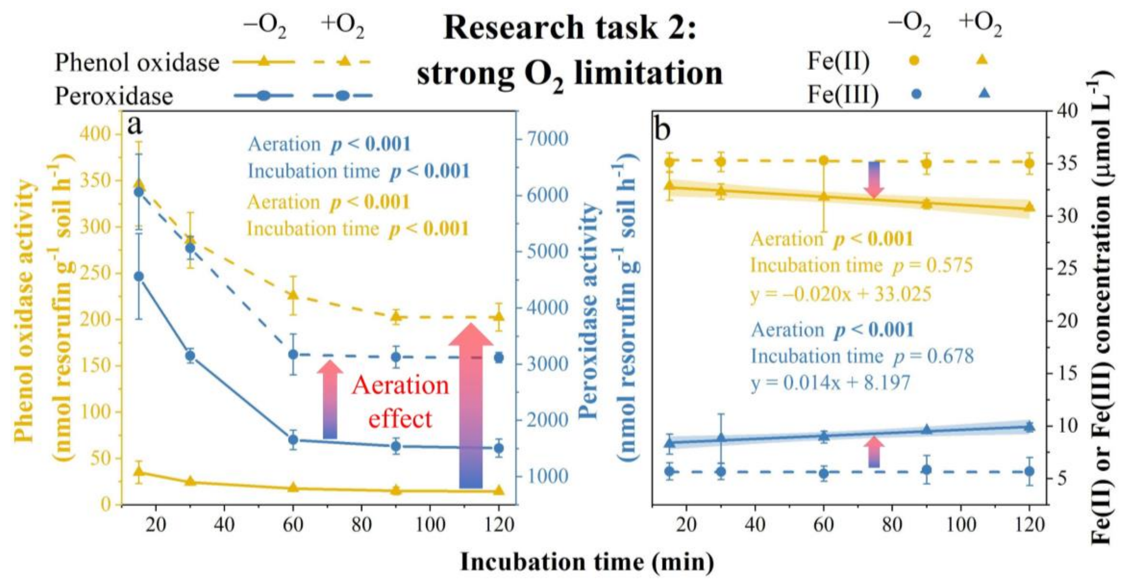
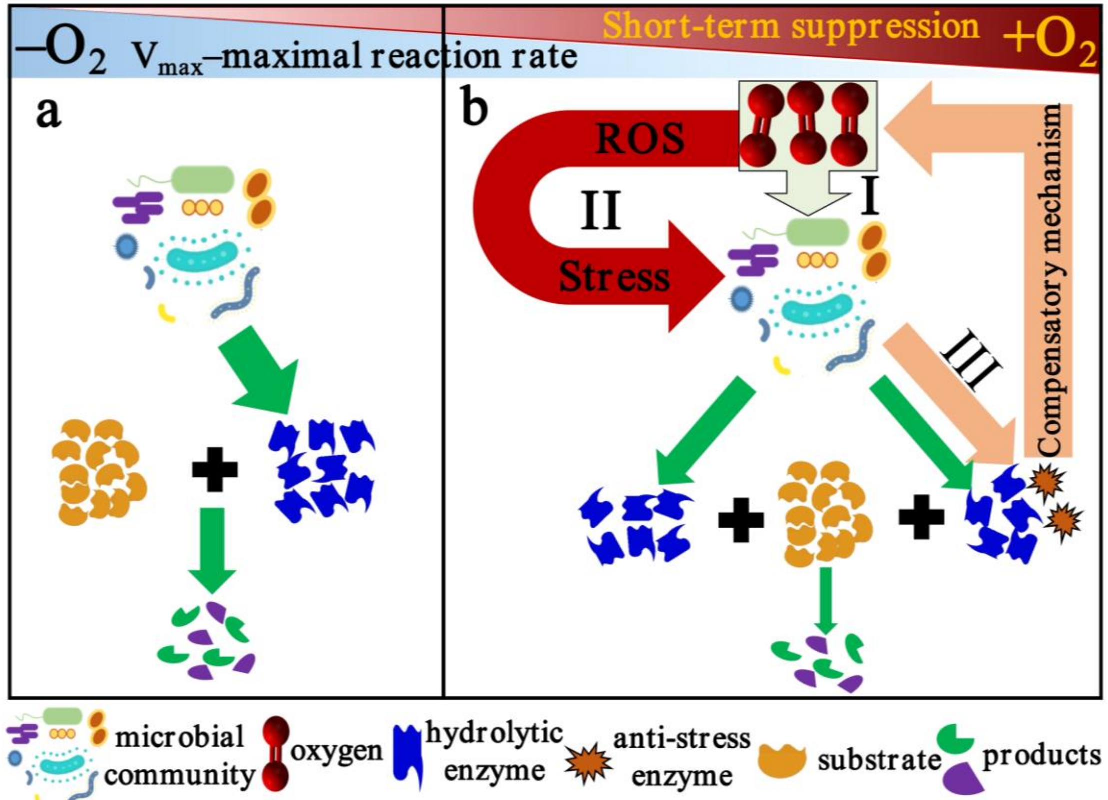



719 **Figure 4**

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721 **Figure 5**